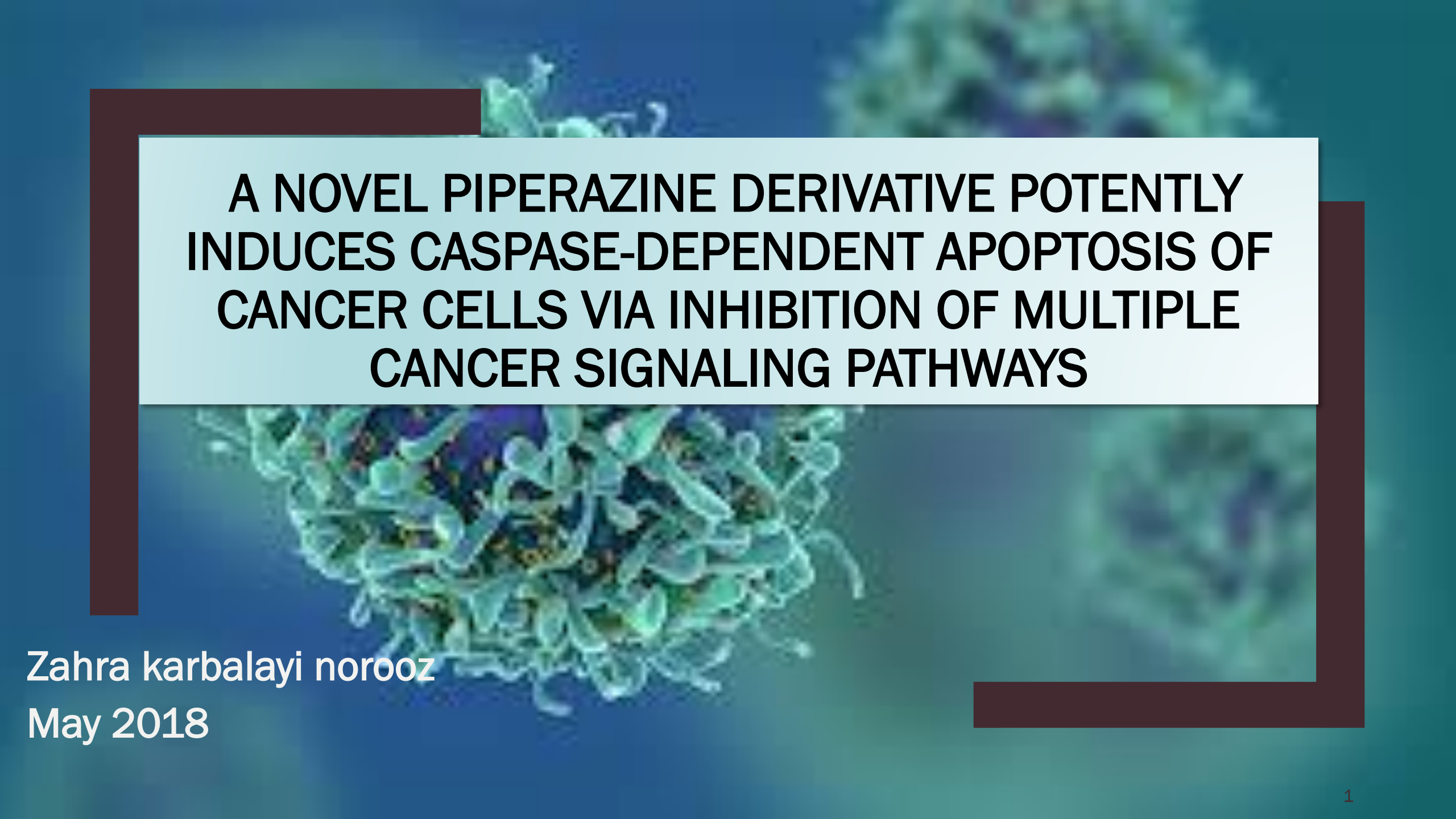
A NOVEL PIPERAZINE DERIVATIVE POTENTLY INDUCES CASPASE-DEPENDENT APOPTOSIS OF CANCER CELLS VIA INHIBITION OF MULTIPLE CANCER SIGNALING PATHWAYS



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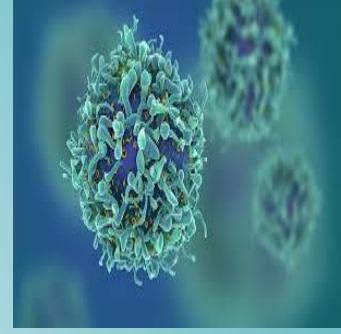

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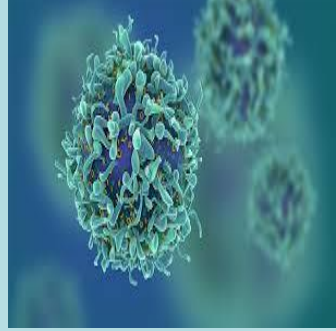
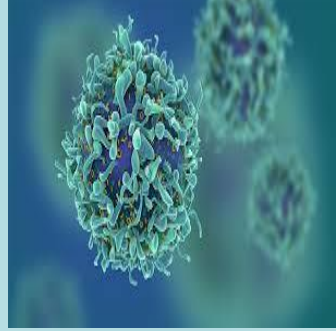
Introduction



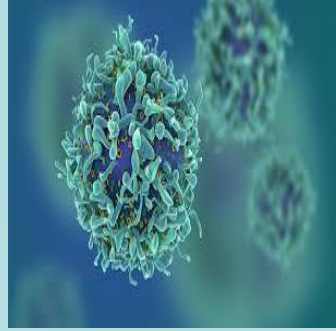
- Cancer is a group of diseases that is characterized by uncontrollable cell growth.
- Cancer accounts for 1 in every 4 deaths in the United States
- Therefore, it is crucial to discover novel anticancer compounds to both prevent and treat cancer.



Introduction

- Previous generations of drugs for cancer treatment were discovered largely from a limited number of chemical entities and these drugs usually target the general cellular machinery related to cell proliferation.
- Such therapies are toxic to not only cancer cells but also normal fast-growing cells.
- The new drug targets may be specifically altered only in cancer cells, allowing development of drugs specially targeted towards cancer cells.
- The overall objective of this study was to identify small molecule compounds synthesized through combinatorial chemistry as candidates for anti-cancer drug development.

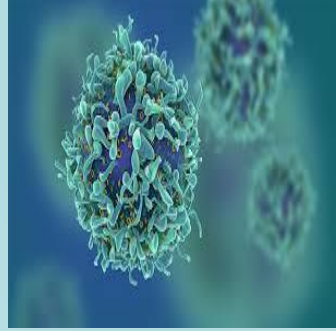
Materials and methods
cell culture:



- **Cancer cell lines**:
  - *K562 (human chronic myelogenous leukemia (CML) cells),*
  - *HeLa (cervical cancer cells),*
  - *AGS (gastric adenocarcinoma cells) were obtained from the American Type Culture Collection (ATCC).*
- K562, HeLa, and AGS cells were cultured in RPMI 1640 medium with 10% Fetal Bovine Serum and 1% antibiotics (PSA).
- Cells were incubated in 5% CO2 at 37°C and were given medium and plate changes as needed.

Materials and methods

Drug library and screening:

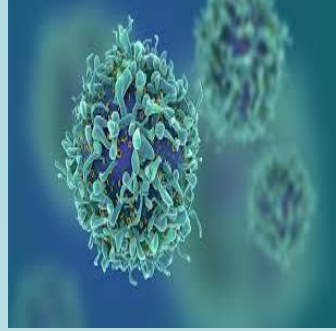


- A library of 2,560 small compounds was purchased.
  - *One-hundred nanoliters of drug was transferred into 100 µl of culture medium in 96-well plates and the final concentration was approximately 10 µM for each drug.*
  - *K562 cells were then added into the wells at a density of* ***3,000 cells/well.***
  - *Plates were incubated for three days at 37°C and 5% CO2.*
  - *Viable cell number was assessed.*
  - *and the optical density (OD) values were recorded.*
- OD value for each experimental well was converted to **cell number** based on the standard curve
- Growth inhibition was calculated by first subtracting the original number of cells seeded (3,000), and then using the following formula:

GI = (control cell number – experimental cell number)/control cell number

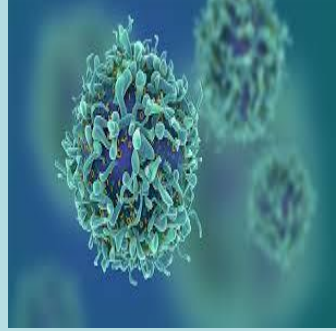
Materials and methods

Evaluation of 50% growth inhibition (GI50) by proliferation assay:



- Cancer cells (K562, HeLa, and AGS) were prepared and diluted to a concentration of 3.5×10^4 cells/mL.
- The center 6 x 10 wells of the 96-well plates were seeded with the cell suspension, totaling 3.5×10^3 cells/well.
- The border wells of the 96-well plates were seeded with autoclaved water to minimize evaporation of the center wells.
- The plates were incubated in 5% CO2 at 37°C overnight, giving ample time for adherent cell lines to attach to the plates.
- Plates were then incubated for another three days.
- estimated the number of viable cells in each well
- The data was then analyzed to construct a GI50 curve and calculate the GI50 value

Materials and methods
Analysis of apoptosis and DNA contents:



- Annexin V is a 35-36 kDa Ca2+ dependent phospholipid-binding protein that has a high affinity for the plasma membrane phospholipid phosphatidylserine (PS), and binds to cells with exposed PS, one of the earliest features of apoptosis.
- Annexin V staining precedes the loss of membrane integrity which accompanies the latest stages of cell death resulting from either apoptotic or necrotic processes
- Therefore, staining with Annexin V is typically used in conjunction with a DNA-binding dye such as 7-amino-actinomycin (7-AAD) which is excluded by viable cells with intact membranes, while the membranes of dead or damaged cells are permeable to 7-AAD.

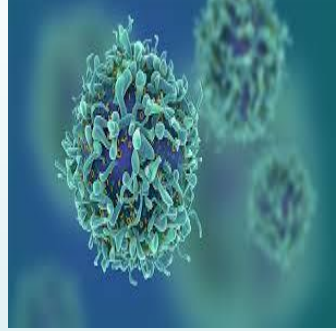
Materials and methods

Analysis of apoptosis and DNA contents:

Analysis of apoptosis :

- K562 cells were prepared and seeded in 24-well at a concentration of 5 x 10^4 cells/well in 2 mL RPMI medium
- Compounds were prepared and added to the wells at a concentration of 5 μM.
- Cells were incubated in 5% CO2 at 37°C for 24 hours.
- Then, cells were collected and washed twice with PBS, centrifuged, and the supernatant was removed.
- Cells were resuspended in 1x Annexin V Binding Buffer
- PE Annexin V and 7-AAD was added to the suspension.
- The suspension was gently mixed and incubated for 15 minutes in the dark at room temperature.
- Samples were analyzed **by flow cytometry**

Materials and methods
Analysis of apoptosis and DNA contents:



- Propidium iodide (PI) is an intercalating agent and a fluorescent molecule that can be used to stain cells.
- PI can be used as a DNA stain for both flow cytometry, to evaluate DNA content in cell cycle analysis and cell viability, and microscopy to visualize DNA containing organelles such as the nucleus.

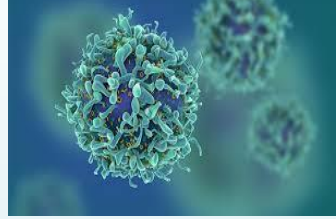
DNA contents analysis:

- K562 cells were also analyzed for DNA contents by propidium iodide (PI) staining.
- Briefly, cells were seeded in 6-well multidishes at a concentration of 1 x 10^6 cells/well in 3 mL of RPMI medium.
- Cells were incubated in 5% CO2 at 37°C. Samples were collected at 24 hour time intervals for 3 days.
- Cell suspensions were transferred to a 5 mL polystyrene tube , centrifuged, and had the supernatant removed and cells were treated with a PI/RNase Staining Buffer and incubated for 15 minutes at room temperature in darkness.
- Cells were analyzed for DNA contents using flow cytometry

Materials and methods
Western blotting:

- Cells were harvested and washed, and lysed in M-PER Mammalian Protein Extraction Reagent supplemented with 1% Halt Protease and Phosphatase Inhibitor Cocktail for 15 minutes.
- Samples were then centrifuged at 13,000 rpm for 10 minutes at 4°C and the supernatant was collected.
- Protein concentration was measured using a Nanodrop 1000 Spectrophotometer
- proteins were separated by SDS-PAGE and transferred to PVDF membranes
- Immuno-positive bands were photographed on X-ray films

Results



High throughput screening of compound library:

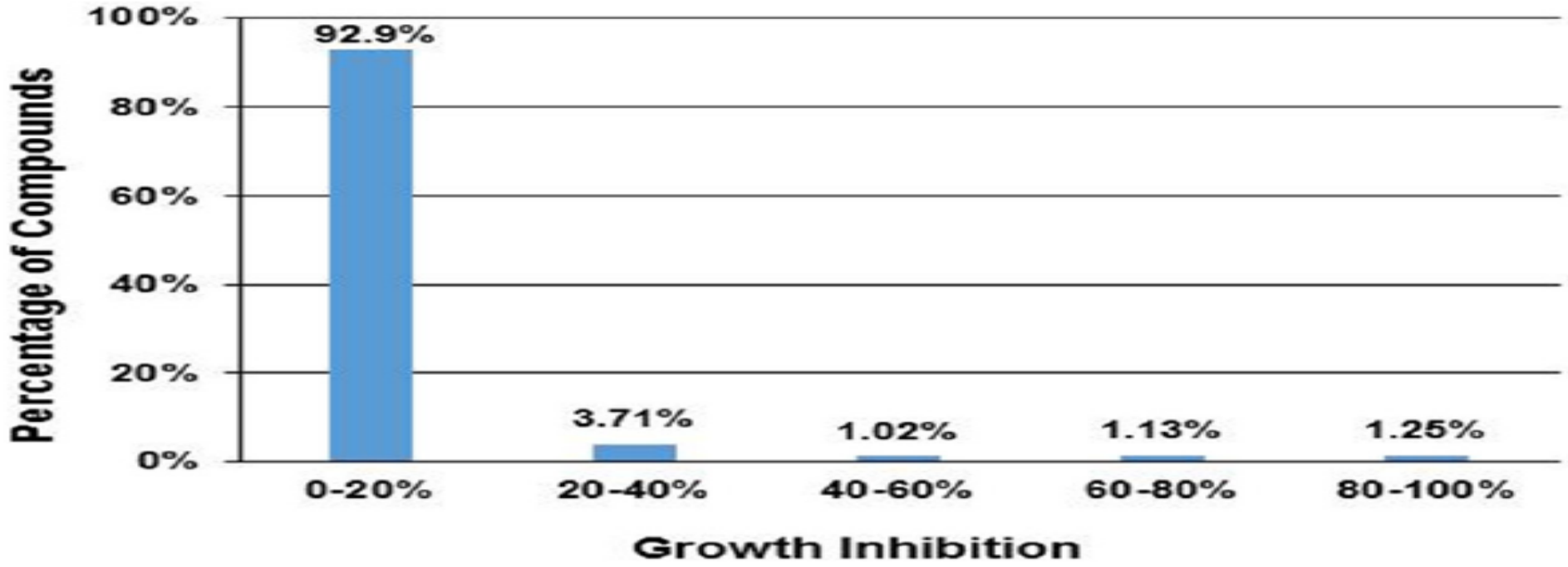
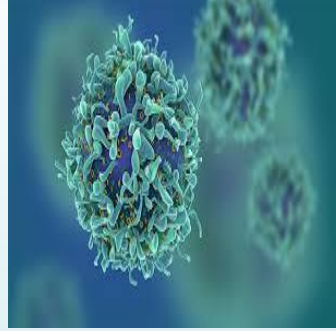
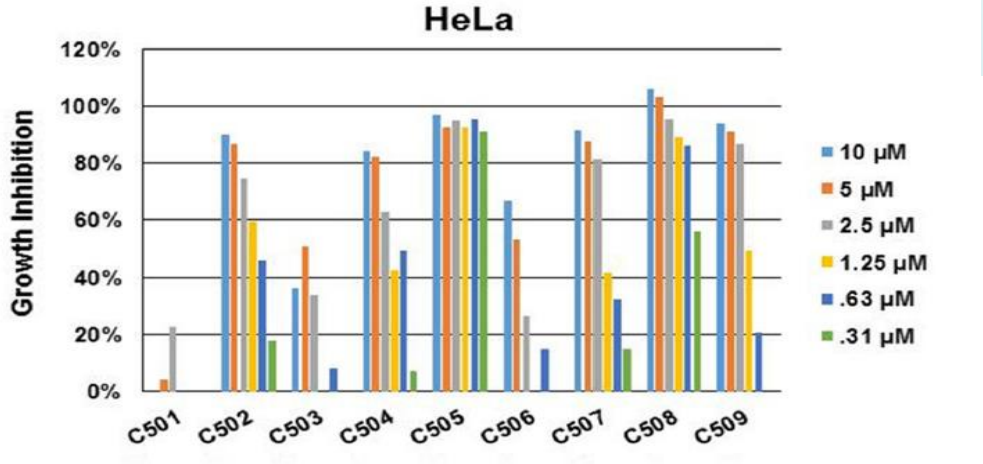


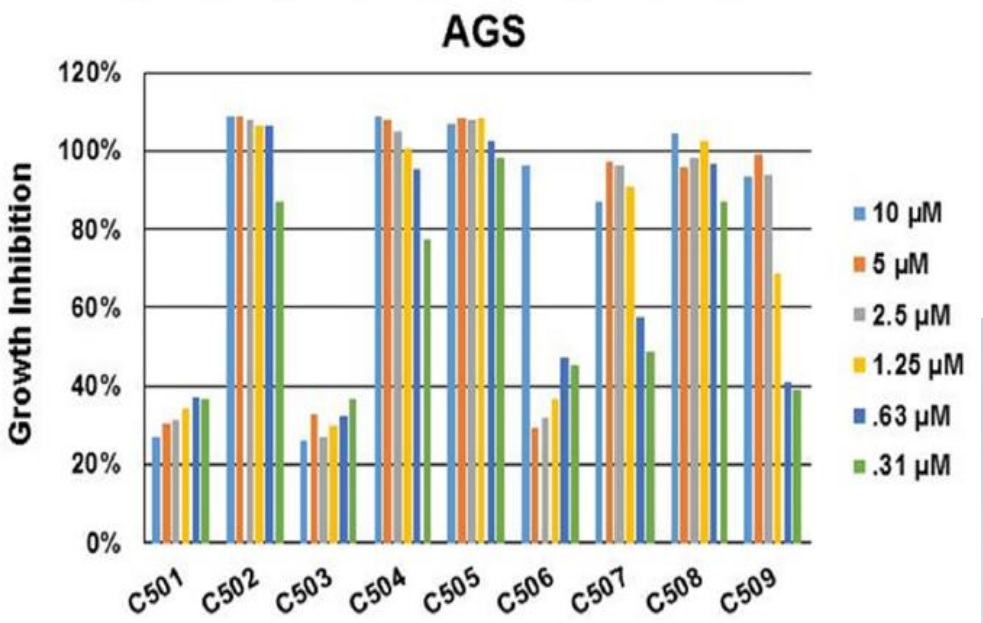
Figure 1. High throughput screening of drug-like compounds for their anti-cancer activity. A total of 2,560 compounds were assayed using a cell proliferation assay with K562 cells. Shown are the percentages of compounds with different ranges of growth inhibition.

Results

Secondary evaluation of nine selected compounds using 3 cancer cell lines:







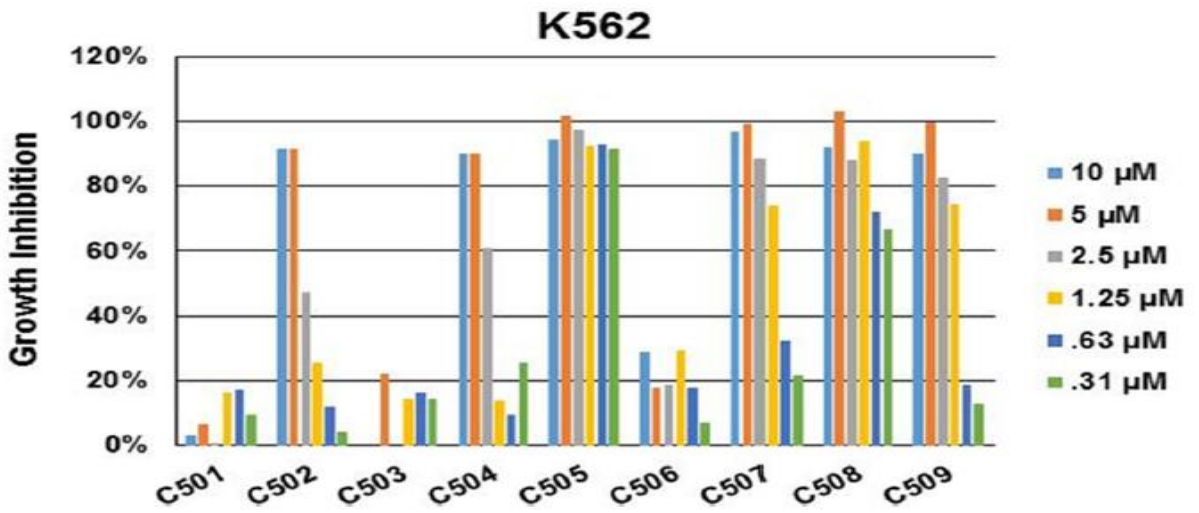
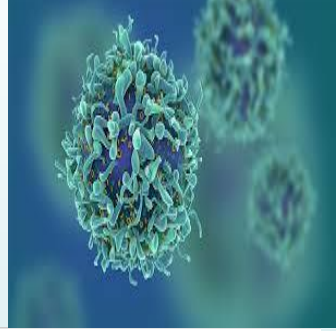


Figure 2. Growth inhibition of nine compounds against three different cancer cell lines. Six different concentrations were tested for each compound. Growth inhibition varies according to compounds, compound concentration, and cell lines. Generally, growth inhibition decreases as compound concentrations decreases.

Results

Determination of GI50 for compound C505:



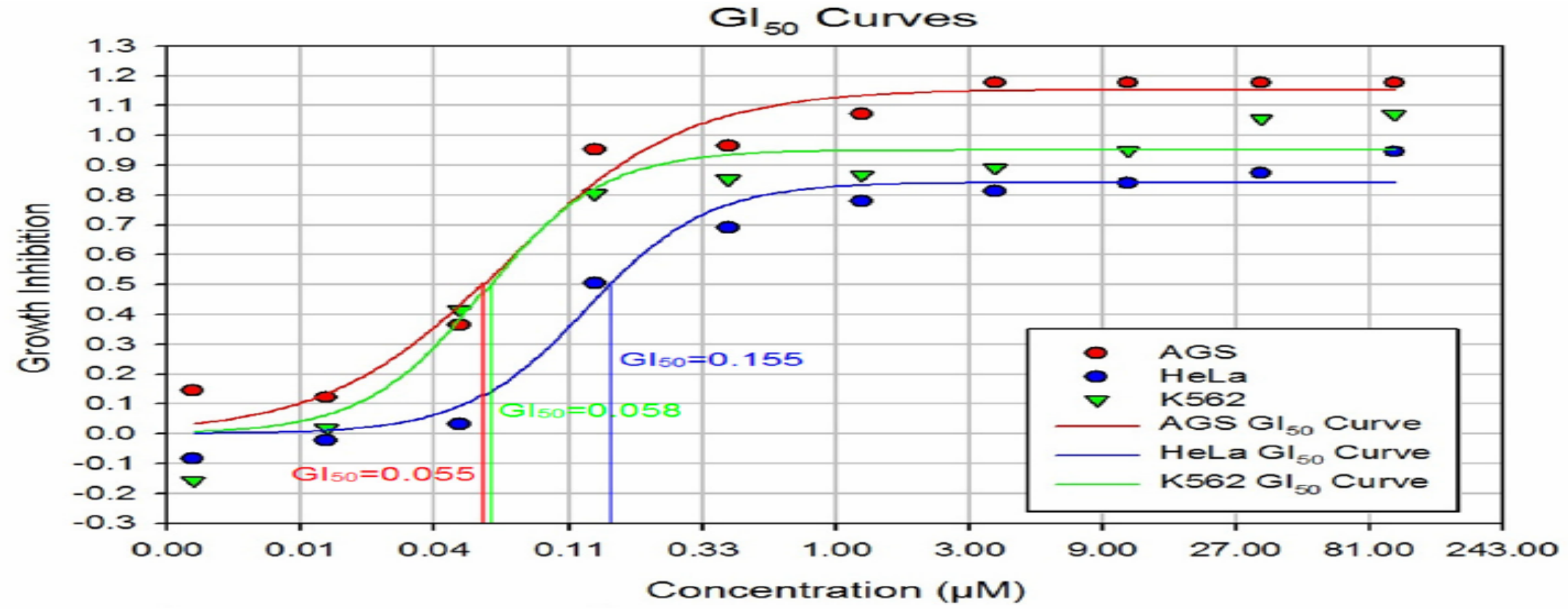
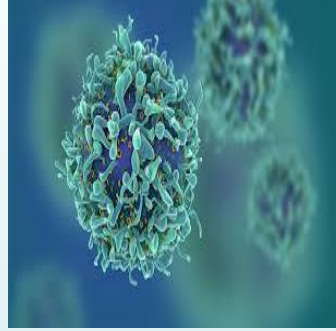


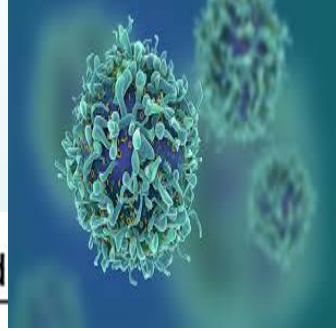
Figure 3. Growth inhibition curves and GI_{50} values for compound C505. Nine different concentrations of C505 were used to treat three different cancer cell lines for 72 hours to establish growth inhibition curves (plots of growth inhibition by compound concentration). The GI curves allow the estimation of GI_{50} value, which is the compound concentration required to achieve 50% inhibition.

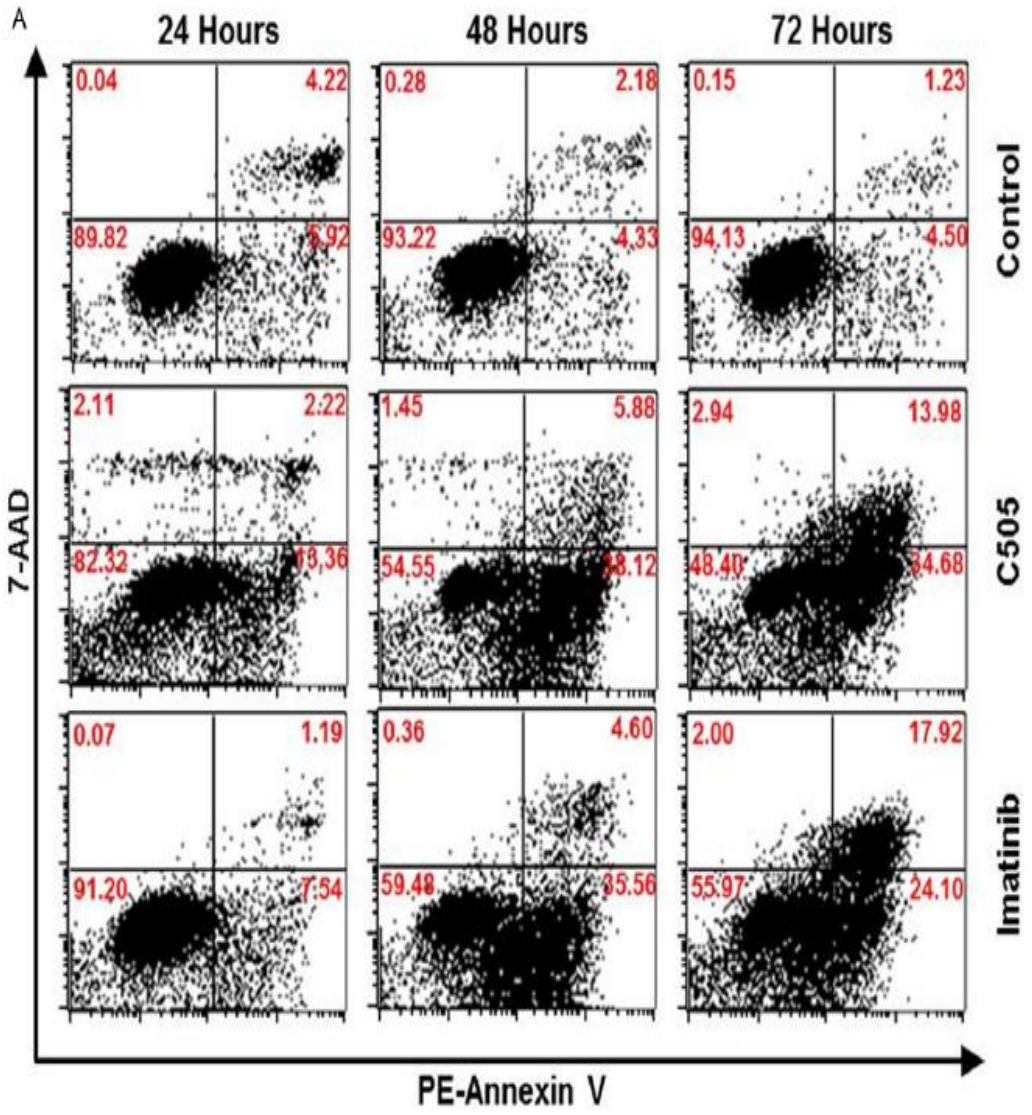
Results

C505 kills cancer cells via induction of apoptosis :



- viable cells are :
  - **7-AAD and Annexin V negative**;
- early apoptotic cells are:
  - **Annexin V positive and 7-AAD negative**,
- while cells in late apoptosis and dead cells are:
  - **both Annexin V and 7-AAD positive.**





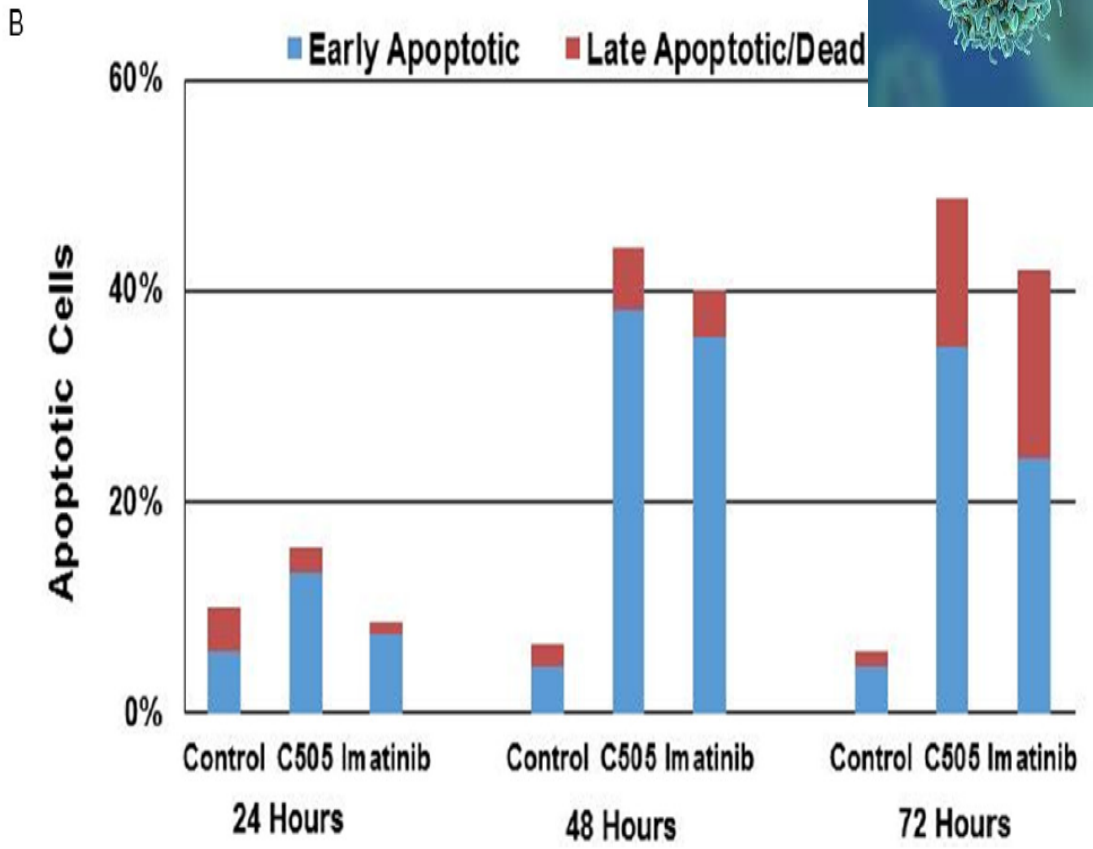


Figure 4. Annexin V and 7-AAD double staining. K562 cells were treated with 5 μM of C505 for 24, 48 and 72 hours before staining. A. Flow cytometry profiles showing staining of cells for Annexin V (x-axis) and 7-AAD (y-axis). The number in each quadrant shows the percentage of cells that are located in that quadrant. Lower left quadrant (double negative) cells are viable cells; lower right quadrant (Annexin V positive and 7-AAD negative) cells are early apoptotic cells; and upper right quadrant (double positive) cells are late apoptotic or dead cells. B. Bar chart presentation of the data shown in **Figure 4A**. Shown are the percentages of early apoptotic cells (blue) and late apoptotic or dead cells (red).

Results

Propidum iodide (PI) staining for apoptosis and cell cycle analysis:

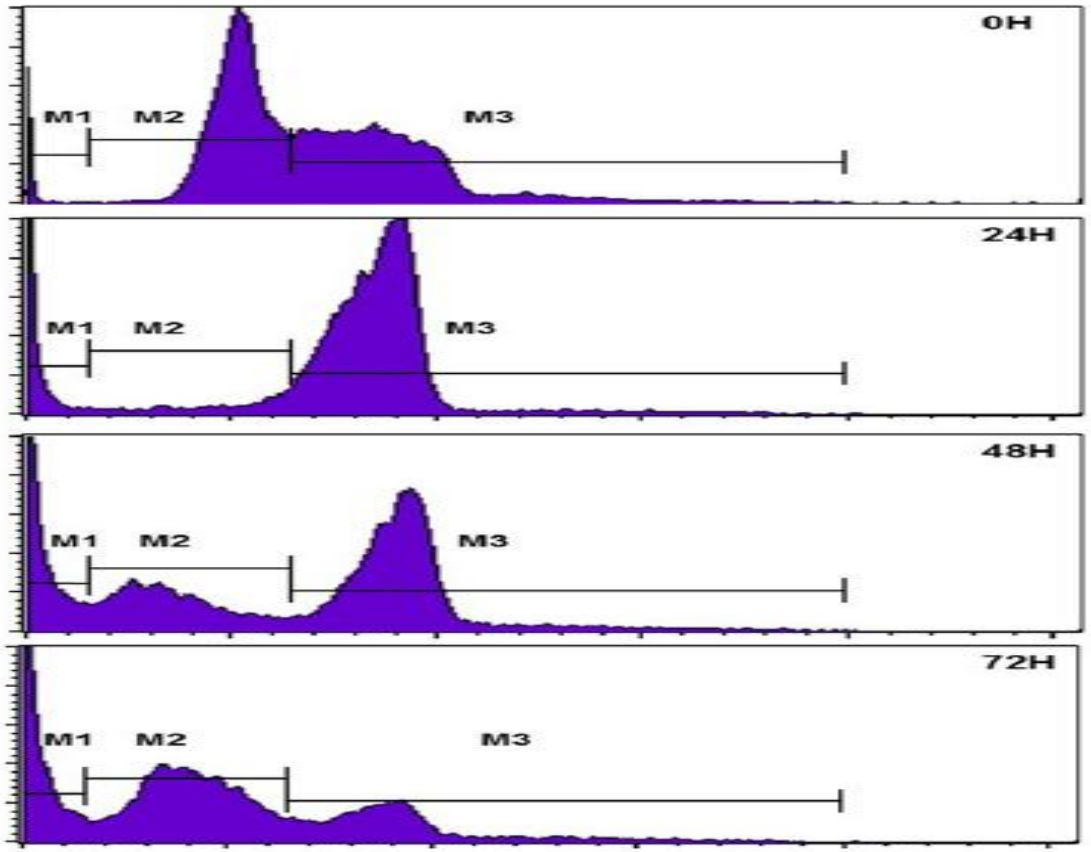
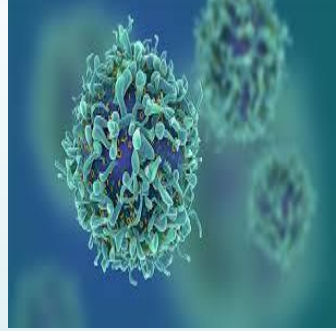
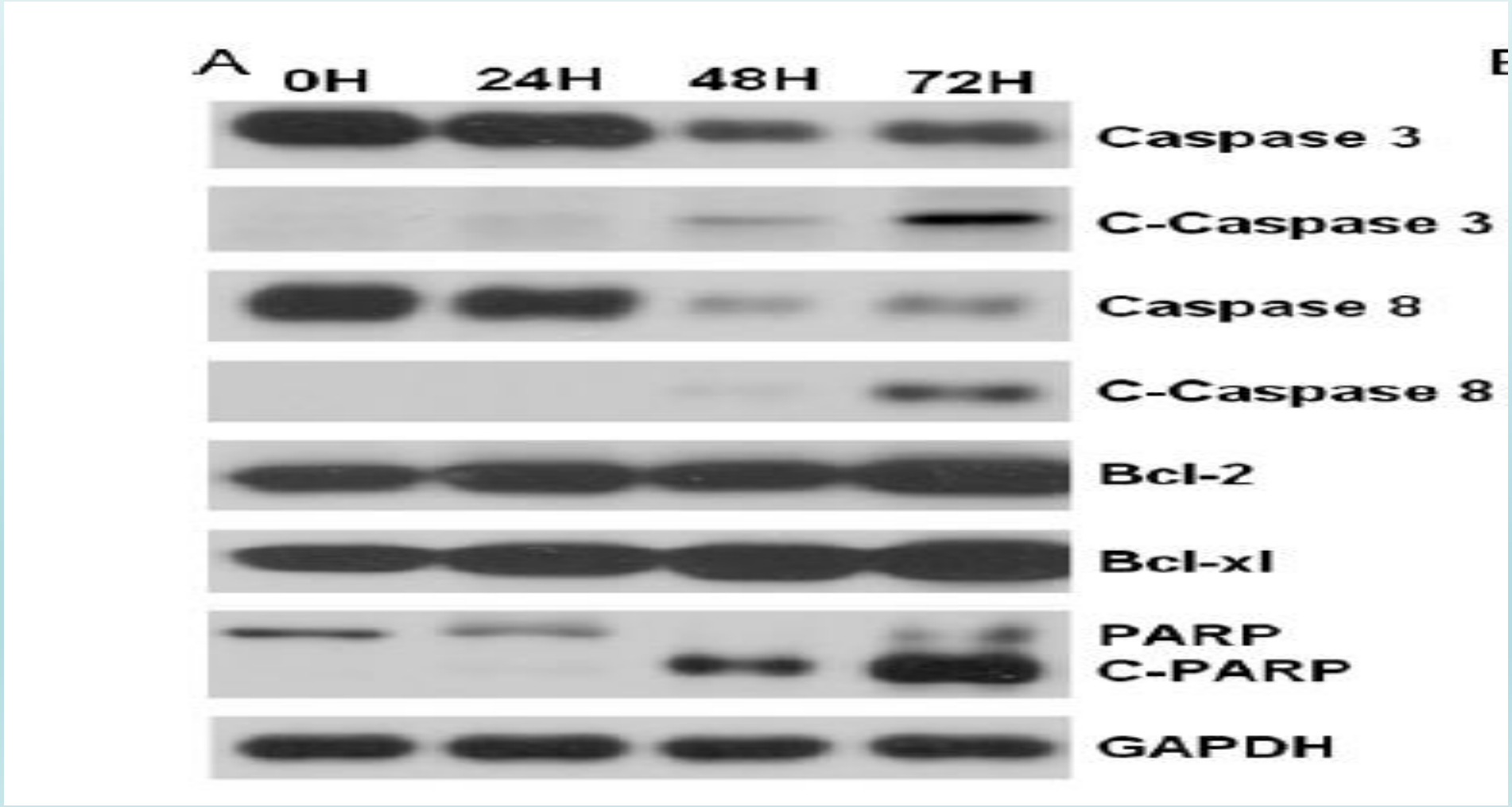


Figure 5. Propidum iodide (PI) staining for apoptosis and cell cycle analysis. K562 cells were treated with 5 μM of C505 for 0, 24, 48 and 72 hours before staining with PI. After treatment, cells were analyzed using flow cytometry. Shown on the left panel are frequencies of cells with increasing PI intensity (or DNA content) from left to right. The cells can generally be classified into three groups corresponding to different cell cycles and viability. Cells in the M1 gate are apoptotic cells (sub G0/G1); cells in the M2 gate are cells at early cell cycle before DNA replication (G0/G1 phase); and cells at the M3 gate are cells at the late stage of cell cycle with active DNA synthesis (S/G2/M). Bar charts on the right panel summarize the percentage of apoptotic cells (M1 gate) and late cell cycle cells (S/G2/M) at different treatment time points.

Results

C505 induces caspase-dependent apoptosis:





Discussion

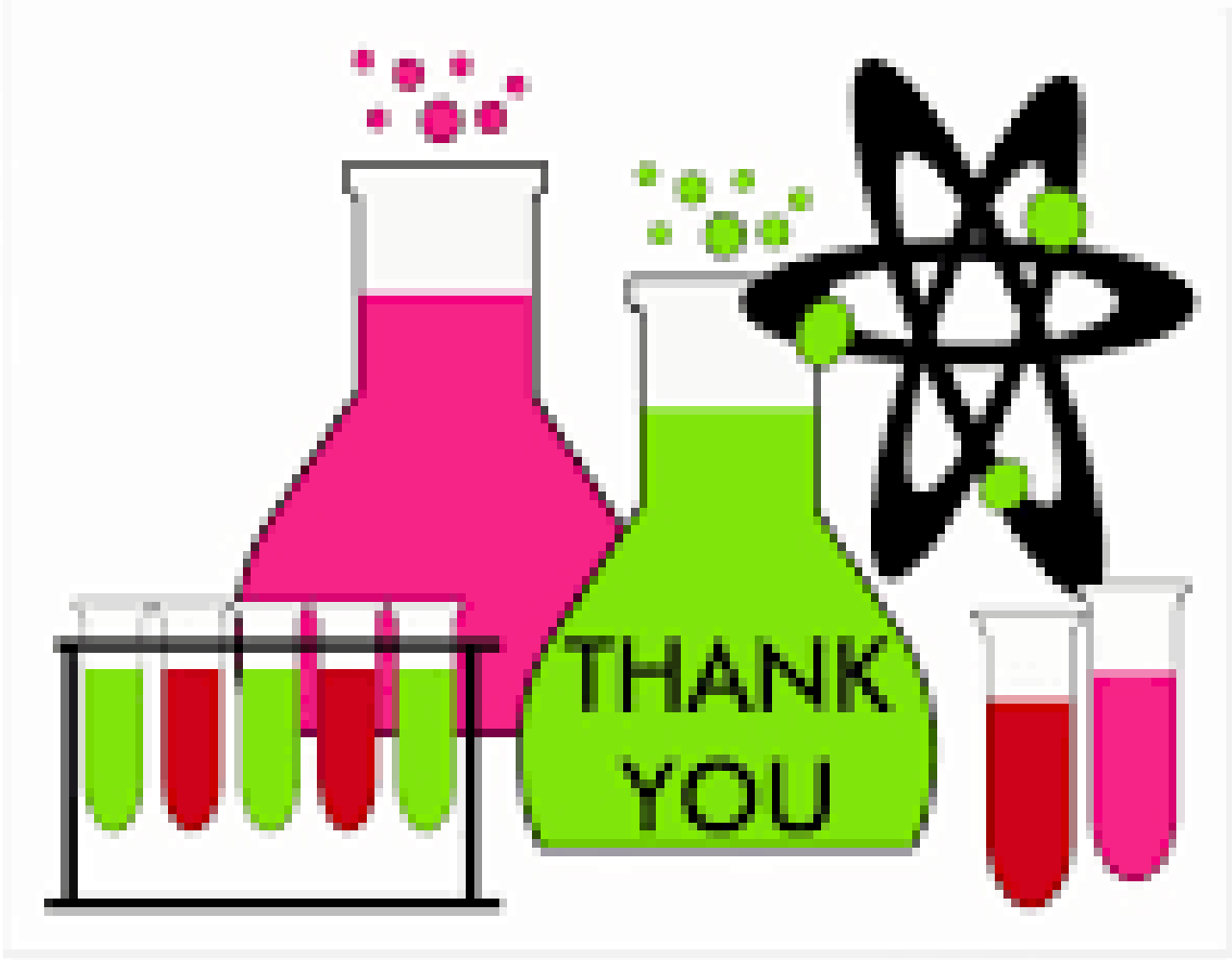
- In this study, we identified one compound with potent anti-cancer activity from a library of 2,560 potential drug compounds.
- The selected compound C505 exhibited toxicity at very low concentrations(GI50 < 0.16 μM) against all three cancer cell lines (K562, HeLa, and AGS) tested.
- C505 is a piperazine-containing compound that was shown in this study for the first time to possess potent anticancer activity.
- The piperazines are a large class of chemical compounds, many of which have important pharmacological properties.
- Many currently notable drugs contain a piperazine ring as part of their molecular structure. Examples include antianginals, antidepressants, antihistamines, antipsychotics, urologicals, and recreational drugs. Indeed, imatinib, which has potent anti-leukemia activity and is used as a positive control drug in this study, is a piperazine derivative
- Given the high potency of C505 and the potentially excellent pharmacological properties, C505 is an excellent candidate that may be further developed into an anticancer drug.

Discussion

- series of experiments was also conducted to determine the cellular and molecular mechanism underlying the killing activity of C505.
- Annexin V/7-AAD staining as well as PI staining indicates that C505 can kill cancer cells via the induction of cell cycle arrest and apoptosis.
- Western blotting analyses suggest that C505-induced apoptosis is caspase-dependent as several caspases including caspase-8 and caspase-3 are activated by C505 treatment

conclusion

In conclusion, we discovered a novel piperazine compound with potent anticancer activity through high throughput screening of a drug-like compound library. This compound effectively inhibits cancer cell proliferation and induces caspase-dependent apoptosis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